Quarterly Progress to:	National Institutes of Health
Contract Monitor:	William Heetderks, Ph.D.
Research Contract:	"Surface Modification for Biocompatibility"
Contract No.:	NS 5-2322 - #9
Principal Investigators:	David C. Martin and K. Sue O'Shea
Date:	April 30, 1997

Overview

This report is a summary of our activities in the ninth quarter of our contract, corresponding to the first quarter of 1997. In this ninth period of activities, we have continued developing and refining our processing characterization techniques. In addition, we have initiated an *in vivo* study of silicon probes coated with the protein polymers in the Guinea Pig CNS. This report provides an overview of the major results to date and discusses our plans for the future. We have been working to (1) characterize protein polymer films, (2) develop assays to evaluate bioactivity of protein polymer films *in vitro*, and (3) evaluate bioactivity and stability of protein polymer films *in vivo*. We also describe our efforts to discuss our work in (4) external communications with the scientific community.

1. Protein Film Deposition, Morphology, and Device Characterization

Progress:

We have made additional progress in characterizing the mechanical properties of these thin film protein polymer films as a function of their morphology. As discussed in the previous progress report, nanoindentation tests were performed to evaluate the elastic and plastic deformation response of thin film polymers on solid substrates. Figure 1 presents a set of load-displacement curves for SLPF films of various morphology deposited onto a silicon substrate. The striking differences in mechanical response clearly reveal the near-surface properties of these thin polymer films on the silicon substrate. The dipped and beaded coating were 2.0 ± 0.5 μm thick and the fibrous coating was 10.0 ± 1.0 μm thick. "Si load" refers to an uncoated silicon substrate. These data show apparent plastic deformation of the fibrous coating at distances away from the silicon substrate. This is consistent with a model in which the indenter tip initially displaces individual fibers laterally, subsequently compressing the fibers as they collide with their neighbors. SEM micrographs, submitted in the last progress report, show the densification of fibers near the center of indentation. Additionally, the load-displacement curve for the fibrous coating shows an increase in stiffness as a function of indenter penetration depth. As the indenter penetrates further into the fibrous coating, the stiffness evidently increases due to increased coating density at the surface of the silicon surface and the increasing influence of the silicon substrate.

In addition to the nanoindentation tests, tensile tests of protein polymer films were conducted. Films of SLPF were electro-spun onto copper support grids and pulled to fracture at a strain rate of 3×10^{-3} mm/sec. SEM micrographs of a SLPF films fractured in tension is shown in Figure 2a with a higher magnification of the fracture surface in Figure 2b. The individual filaments of protein polymer fail in a manner which indicates little evidence for local plastic deformation. Close examination of the micrographs indicate that the crack propagates along an irregular path as the web of SLPF filaments fracture. This

Figure 2a.

Figure 2b.

data indicates that the fibrous morphology is successful in providing an irregular pathway for crack formation. When these films are placed in-vivo, it is likely that cells from the CNS will help to bridge the gaps between filaments, thus providing a microstructure which simulates the natural tissue and promotes toughness of the film.

We have recently been able to make a correlation between our impedance spectroscopy measurements, which probe the dynamics of carrier transport as a function of time frequency, and our atomic force microscopy measurements, which provide information on surface morphology as a function of spatial frequency. We have found that we observe self-similar behavior in the impedance dynamics over a range of time frequencies. Also, our microstructural investigations indicate that our surface morphologies show a self-similar behavior over a range of spatial frequencies. By combining information from these two experiments we have appear to have been able to obtain insight about the mechanisms of carrier transport. In particular, we have been able to estimate a diffusion coefficient D on the order of ~10^{-9} cm^2/sec for the critical transport process at the surface. Additional details about these results and our analysis will be provided in the next quarter's report.

Plans:

Our nanoindentation experiments have also shown that it is feasible to measure the mechanical property variations of the protein polymer films as function of distance from the free surface by examining the mechanical response as a function of probe indentation. Our analyses to date show that the film is providing the hypothesized intermediate gradient in stiffness that should prove useful to promote matching between the mechanical properties of the rigid silicon substrate and the flexible tissue of the CNS. We have also found that the gradients in stiffness are more gentle in the electrospun fibrous films than in the continuous films, as is also confirmed by the data discussed in this report. Our quantitative analyses of the film stiffness as a function of thickness are ongoing and will be discussed in more detail in the next quarter's report.

2. Bioactivity of Protein Polymer Films *in vitro*

Progress:

We have continued to develop and refine our stamping technique for creating patterns of protein films. A device was fabricated to align the stamps with the glass substrates. The use of this device has increased production of useable substrates. Substrates stamped with SLPF, fibronectin, laminin, and hydrophobic and hydrophillic silanes followed by incubation with fibronectin and laminin have been produced. In preliminary studies, precoating of nitrocellulose, described in the previous progress report, appeared to debond form the glass substrate after 24-48 hours in aqueous media. The use of nitrocellulose is currently being re-evaluated as a precoating. In one set of experiments, substrates were seeded with Neuro-2A cells at three different cell densities: $3x10^4$, $3x10^5$, $3x10^6$ cells per milliliter. The cells were confined to the center of the substrate for 1-2 days, then allowed to migrate freely. The optimal cell density was one in which the majority of cells were able to adhere to the available surface area but "crowded" enough to encourage migration away from the center of the substrate. This optimal cell density for our test conditions was between 10^5 to 10^6 cells per milliliter. Preliminary studies have also shown that cell grown in medium were able to adhere to bare glass substrate after 24 hours. We are currently investigating the use of cells grown in serum-free medium.

A proposal submitted to the Cornell Nanofabrication Lab to manufacture patterned substrates was approved last month. In addition, a fellowship was awarded to offset user fees at the facility. Dr. Libby Louie will be traveling to Cornell in the next few weeks to start fabricating patterned microstamps and substrates.

An assay to quantify the adhesion of cells to various substrates was developed. Cells were plated onto substrates for 30 minutes, then agitated 5 minutes on a orbital shaker at 120 RPM. Preliminary analyses indicate the the number of cells per unit area adhered is similar for an 80% coverage beaded coating, a 80% coverage fibrous coating, and a 100% coverage continuous coating (Figure 3).

Plans:

Patterned substrates produced at the Cornell Nanofabrication Lab will be plated with Neuro-2A cells grown in serum-free medium an evaluated at various time points. Assays to quantify extent of adhesion and morphology of cellular structure will be continued to be developed and refined.

3. Bioactivity of Protein Polymer Films *in vivo*

Progress and Plans:

An investigation to study the histological effect of coated silicon probes has been initiated. Three-dimensional arrays of silicon probes are in the process of being coated with either SLPF, SLPL, or SELP (n= 4 each). Uncoated probes will be used as controls (n=4). The probes will be implanted in the cortex of the guinea pig and evaluated at three weeks post-operative. Each animal will have one multiple-shanked probe inserted into each brain hemisphere, for two probes total per animal. Histological techniques developed to evaluate the longitudinal interface between the probe and tissue (discussed in the last progress report) are currently being refined.

4. Outside communications

An abstract was written and submitted to the Society for Neuroscience titled "Glial and Neuronal Cell Response to Substrates Patterned with Multiple Native and Synthetic ECM Proteins", by L. K. Louie, D. C. Martin, K. S. O'Shea, and M. Hortsch. A copy of this abstract is enclosed.

Christopher J. Buchko successfully passed his Ph.D. data review and is currently writing his Ph.D. Dissertation.

Shenkarram A. Athreya is actively writing up the results of his impedance spectroscopy measurements on protein polymer coated silicon probes.

A second request for coated probes for implantation into the toadfish was received from Dr. Allen Mensinger in the Department of Otolaryngology at Washington University Medical School, and we are preparing samples for sending back for his continued evaluation.

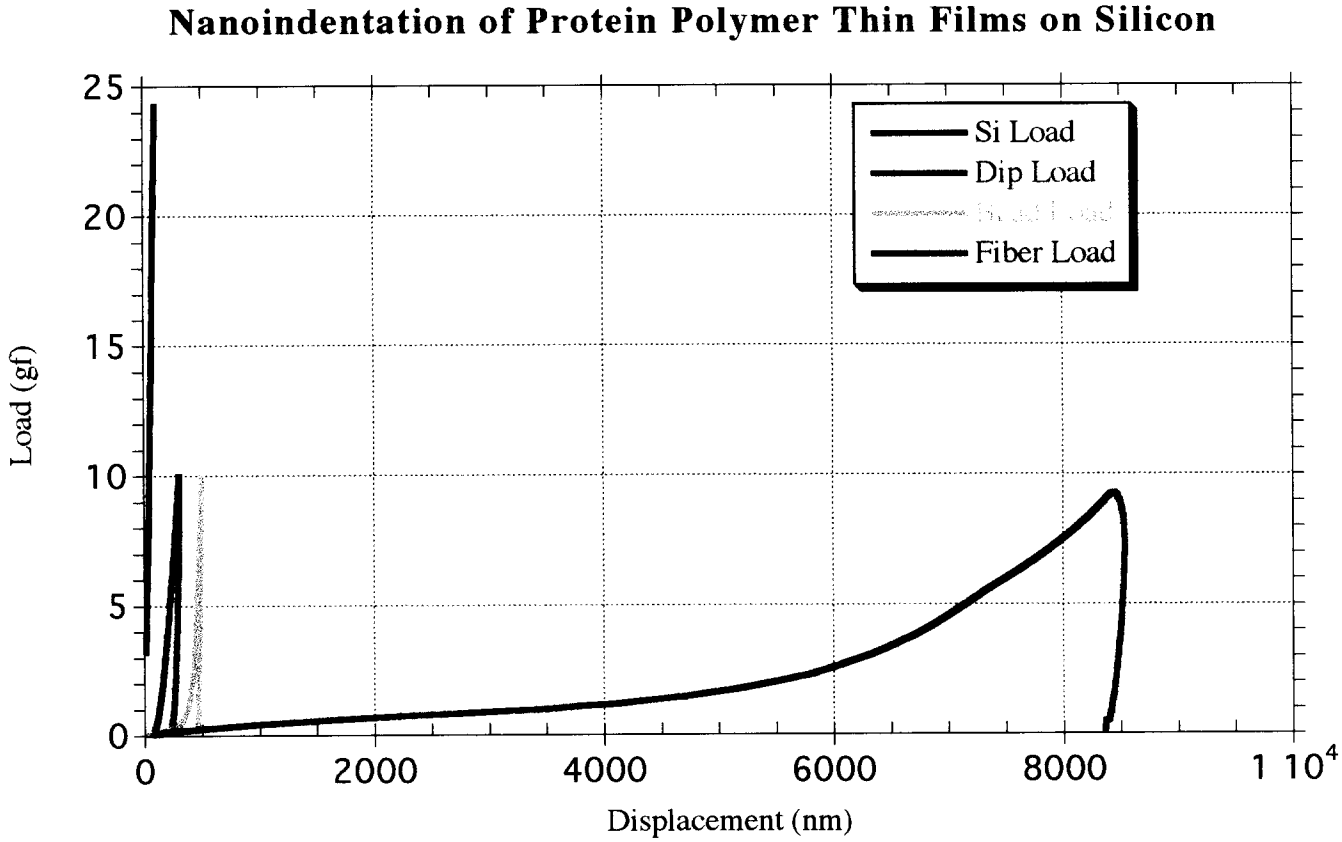
Nanoindentation of Protein Polymer Thin Films on Silicon
Si Load
Dip Load
Fiber Load
Load (gf)
Displacement (nm)
0
5
10
15
20
25
0
2000
4000
6000
8000
1 10^4

Figure 1.

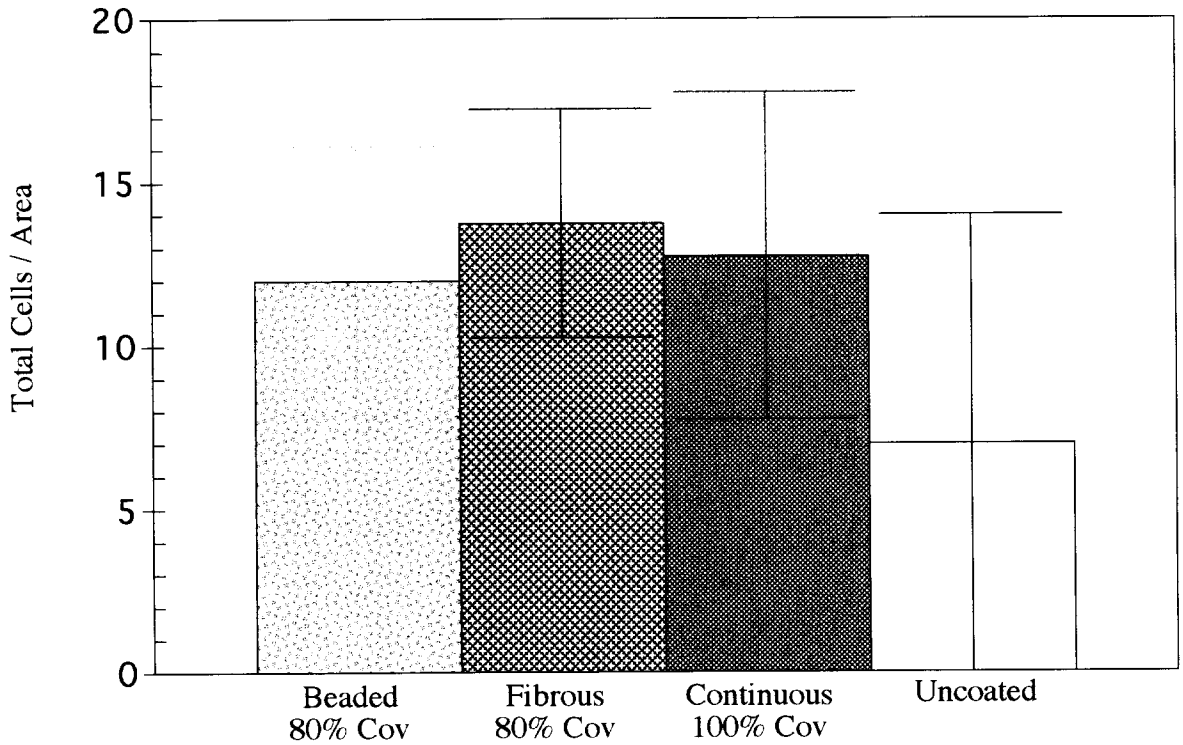
Total Cells / Area
20
15
10
5
0
Beaded
80% Cov
Fibrous
80% Cov
Continuous
100% Cov
Uncoated

Figure 3.

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Libby K. Louie
Anatomy & Cell Biology
4811 MSII
Univ of Michigan Med School
Ann Arbor, MI Fax: (313) 763-1166
Office: (313) 647-3990 Home: (313) 741-0619
E-mail: llouie@engin.umich.edu

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GLIAL AND NEURONAL CELL RESPONSE TO SUBSTRATES PATTERNED WITH MULTIPLE NATIVE AND SYNTHETIC ECM PROTEINS.

L.K. Louie[1,2], D.C. Martin[1], K.S. O'Shea[2], SPON: M. Hortsch*[,2]. Depts. of Materials Science and Engineering[1], and Anatomy & Cell Biology[2], University of Michigan, Ann Arbor, MI 48109

Extracellular matrix (ECM) proteins are important in both the development and response to injury of the nervous system. In the current investigation, spatially resolved patterns of native proteins (laminin, fibronectin) and synthetic proteins with cell binding motifs incorporated into the backbone of the biopolymer e.g., the RGD sequence from fibronectin (SLPF), IKVAV from the laminin (SLPL), and VPGVG repeat sequence of elastin (SELP) (products of Protein Polymer Technologies, Inc., San Diego), were adsorbed onto substrates. Patterns were constructed such that cells were given the opportunity to choose between different proteins. Neuro-2A and neonatal Schwann cells grown in serum-free medium were plated onto the substrate and evaluated at 30 minutes, 1, 2, and 24 hours. The length of neurite outgrowth, rate of outgrowth down a particular protein path and morphology of the cytoskeleton and focal adhesions were evaluated.

A reproducible method to produce substrates patterned with more than one protein was developed. The morphology and neurite outgrowth of the cells appeared to depend on the underlying protein pattern suggesting that the cells were able to recognize and modulate their behavior based on substrate characteristics.

Supported by NIH NINDS Contract No. NO1-NS-5-2322.

Key Words: (see instructions p. 4)

1. Fibronectin
2. Laminin
3. neurite outgrowth
4. adhesion

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